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Stability of RNA isolated from human trabecular bone at post-mortem and surgery

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Abstract

To determine the reliability of gene expression studies in human post-mortem bone, it is important to evaluate the stability of RNA isolated from such tissues as a function of the post-mortem interval. The stability of total RNA and bone-specific mRNA species was examined in bone samples obtained from routine autopsies and at surgery. The optimal temperature for any storage and transport of the bone before RNA isolation was shown to be 4 °C, and RT-PCR analysis is the preferred technique for the analysis of gene expression in post-mortem bone as it tolerates partial RNA degradation. For gene expression studies in bone, post-mortem cases, with a post-mortem interval of less than 48 h, should be selected, and the time that bone is stored after retrieval at autopsy or surgery should be kept to a minimum. Overall, our findings indicate that with appropriate storage and handling, RNA can be reliably isolated from human bone obtained at post-mortem and surgery to study ex vivo the pattern of gene expression in healthy individuals and in patients with musculoskeletal diseases such as osteoporosis and osteoarthritis.

Keywords: Bone; mRNA; Post-mortem; RNA stability; Surgery

1. Introduction

Extensive knowledge of systemic and local regulation of bone remodelling has been developed from studies using in vitro cell culture systems and animal studies (reviewed in [1–5]). However, there is limited information regarding the pattern of gene expression corresponding to specific molecules with regulatory roles in bone turnover in normal human bone, or the role of these molecules in skeletal disease. The advantage of investigating the mRNA expression of skeletally active genes in the local human bone microenvironment over cell culture techniques is that paracrine factors and mediators of bone turnover can be analysed with their local regulatory mechanisms intact. Lin et al. [6] emphasized that the analysis of mRNA expression patterns in human tissues can provide significant insight into the spatio-temporal activities of gene transcription in a tissue, and further provide important information on physiology and pathology at a molecular level. Insight may be gained from changes in mRNA levels in bone tissues specifically affected by disease; for example, skeletal sites such as the bone adjacent to an articular joint with osteoarthritis (OA), where the structural parameters and indices of trabecular bone turnover are known [7,8].

Human bone from individuals with skeletal disease, such as OA, rheumatoid arthritis, or fragility fracture, is readily available from joint replacement surgery. However, it is rarely possible to obtain control site-matched tissues from individuals without bone disease and therefore post-mortem bone is a valuable resource. However, the disadvantage of using post-mortem tissues for molecular studies is the time

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interval between death and the post-mortem examination. This post-mortem interval (PMI) is variable and often uncontrollably delayed and the stability of RNA isolated from human post-mortem skeletal tissues is unknown. Recovering intact RNA from post-mortem tissues, specifically bone, may be compromised by the activity of both targeted and generalised ribonuclease enzymes (RNases) that degrade mRNA molecules. Therefore, to determine the reliability of gene expression studies in post-mortem bone, it is important to evaluate the stability of total RNA and specific mRNA species isolated from bone tissues, as a function of the PMI.

The aims of this study were to investigate the post-mortem conditions required to isolate undegraded total RNA from human post-mortem trabecular bone, and to examine the influence of storage conditions on the stability of total RNA and specific mRNA species.

2. Materials and methods

2.1. Human post-mortem bone tissue

2.1.1. Post-mortem case profiles

Proximal femurs and iliac crest samples were obtained from 18 routine autopsies, comprising 10 women aged 43–83 years and 8 men aged 44–81 years, performed at the Royal Adelaide Hospital. Informed consent was obtained from next-of-kin for the collection of these specimens, with the approval of the Royal Adelaide Hospital Human Ethics Committee. Details of age at death and cause of death are shown in Table 1. Bone samples taken from the proximal femur or iliac crest were stored at 4 °C for between 0 and 30 h before RNA isolation.

2.1.2. Sampling of trabecular bone from the iliac crest and proximal femur

Trabecular bone was sampled from the intertrochanteric region of the proximal femur, from the femoral neck, and for a subset of cases, from the iliac crest at a point 3 cm posterior to the antero-superior iliac spine. Immediately after removal from storage at 4 °C (refer above), each proximal femur was sectioned in the coronal plane using a band saw that had been cleaned with DEPC-treated water. This allowed access to trabecular bone for sampling from the femoral neck and intertrochanteric skeletal regions, which are enclosed within the femoral cortex. The iliac crest bone wedge samples were bisected longitudinally to access the trabecular bone enclosed within the cortical shell. Using sterile bone cutters, the trabecular bone was sampled as small fragments from the femoral neck and intertrochanteric regions from an approximately 1.5 × 1.0 cm^2 area to a specimen depth of 0.5 cm. Samples were also taken from the iliac crest from an approximate 1.0 × 1.0 cm^2 area, to a specimen depth of 0.5 cm. The trabecular bone fragments were rinsed briefly in DEPC-treated water and then immersed in RNA lysis buffer (4 M guanidine isothiocyanate solution; 2 ml/250 mg wet weight).

Table 1
Profiles of post-mortem cases examined

Case	Age (years)	Gender	Skeletal regions	Cause of death
A1	50	Male	FN, IT	Chronic renal failure
A2	61	Female	IC, IT	Non-Hodgkin's lymphoma
A3	60	Male	IT	Pneumonia
A4	64	Male	FN, IT	Sepsis
A5	50	Male	FN, IT	Liver failure
A6	57	Female	IC, IT	CML/sepsis
A7	43	Female	IT	Liver failure
A8	72	Female	IT	Respiratory failure/ pulmonary oedema
A9	83	Female	FN, IT	Cardiac arrest
A10	83	Female	FN, IT	Complications post hip surgery
A11	71	Male	FN, IT	Lupus erythematosus
A12	73	Male	IT	Cerebrovascular disease
A13	68	Female	IT	Sepsis
A14	75	Female	IT	Pneumonia
A15	44	Male	IT	Sepsis/pneumonia
A16	68	Female	IT	Post CABG
A17	81	Male	IT	GI haemorrhage
A18[a]	57	Female	IT	Abdominal sepsis

A, autopsy case; FN, femoral neck; IC, iliac crest; IT, intertrochanteric region of the proximal femur. CABG, coronary artery bypass surgery; CML, chronic myelogenous leukaemia; GI, gastrointestinal.
[a] Case used for time-course analysis.

The minimum practical PMI before harvesting of bone was found to be 24 h. To attempt to replicate the effect of longer PMIs on RNA degradation, the integrity of specific mRNA species was investigated after storage at 4 °C of trabecular bone. A sample from the intertrochanteric region of one post-mortem case (A18), with a PMI of 24 h, was further divided into four samples of equivalent tissue weight (approximately 250 mg wet weight of trabecular bone tissue, including bone marrow). Total RNA was isolated immediately from one portion to represent a 24-h PMI time-point. The remaining three samples were kept in sterile RNase-free 0.85% saline at 4 °C, and RNA was sequentially isolated at 24-h intervals up to 96 h.

2.2. Human surgical bone tissue

2.2.1. Surgical case profiles

Trabecular bone samples obtained at surgery were used to investigate the effect of storage temperature and storage time on the integrity of total RNA and specific mRNA species in human bone. The samples were obtained from seven patients (four women, three men, aged 70.3 ± 11.5 years) undergoing total hip arthroplasty surgery for either severe OA of the hip or subcapital femoral neck fracture. Patient details are shown in Table 2. Informed consent was obtained prior to the collection of these specimens, with approval from the Royal Adelaide Hospital Human Ethics Committee. Trabecular

Table 2
Patient details for surgical specimens

Case	Age (years)	Gender	Skeletal regions	Skeletal disease
S1	71	Female	PT	Early knee OA
S2	49	Female	IT	Severe hip OA
S3	69	Male	IT	Severe hip OA
S4	77	Male	IT	OP/#NOF
S5	81	Female	FN, IT	OP/#NOF
S6[a]	80	Female	IT	Severe hip OA
S7[b]	62	Male	IT	Severe hip OA
S8[c]	74	Female	IT	OP/#NOF

S, surgical case; FN, femoral neck; IT, intertrochanteric region of the proximal femur; PT, proximal tibia. OA, osteoarthritis; OP, osteoporosis; #NOF, subcapital femoral neck fracture.

[a] Case used for time-course analysis; bone tissue was stored at 4 °C.

[b] Case used for time-course analysis; bone tissue was stored at room temperature.

[c] Case used for time-course analysis; bone tissue was stored at 37 °C.

bone from the proximal tibia was obtained from patient S1, who was undergoing a tibial osteotomy.

2.2.2. Sampling of trabecular bone from the proximal femur and tibia

During total hip arthroplasty surgery, a 10-mm internal diameter tube saw was used to take trabecular bone core biopsies of the intertrochanteric region, in line with the femoral medullary canal [9]. In addition, trabecular bone was sampled from the femoral neck region of patient S5 and trabecular bone was obtained from the proximal tibia of patient S1 (Table 2). The fresh surgical bone samples were placed in cold sterile RNase-free 0.85% saline and transported directly to the laboratory. Samples were separated into small fragments using sterile bone cutters and/or a sterile scalpel blade. The trabecular bone fragments were rinsed briefly in DEPC-treated water and then immersed in RNA lysis buffer, as above.

Trabecular bone from the intertrochanteric region of patients S6, S7, and S8 (Table 2) was used to investigate the effect of storage temperature and storage time on the integrity of total RNA and specific mRNA species. Tube saw core biopsies 10 mm in diameter and 4, 5.5, and 4.5 cm in length, respectively, were divided into eight pieces of approximately equivalent tissue weight. Total RNA was isolated from the bone sampled from one end of the core biopsies, to represent a 0-h time-point. The remaining seven bone samples were kept in sterile RNase-free 0.85% saline at 4 °C or room temperature (22 ± 1 °C) or 37 °C, for cases S6, S7, and S8, respectively, and RNA was isolated consecutively at 2, 4, 8, 16, 24, 48, and 72 h.

2.3. Isolation of total RNA from human post-mortem and surgical trabecular bone

Total RNA was extracted from the post-mortem and surgical trabecular bone using an adaptation of the protocol described by Davey et al. [10] for human trabecular bone [11], which takes into account the large amount of extracellular matrix and mineral present in skeletal tissue. Briefly, the trabecular bone fragments (2 ml/250 mg wet weight) in a 4 M guanidine isothiocyanate solution (25 mM sodium citrate, pH 7.0, 0.5% sarkosyl, 0.1 M β-mercaptoethanol) were homogenized using an Ultra-Turrax (TP 18/10; Janke and Kunkel, Staufen, Germany). The homogenized sample was clarified by centrifugation (1000 × *g* for 5 min) to separate the homogenate from the insoluble bone mineral, vortexed in 0.1 volume of 2 M sodium acetate, pH 4.0, and then extracted with one volume of phenol and 0.2 volume of chloroform/isoamyl alcohol (49:1). Total RNA was precipitated with isopropanol, resuspended in 1 × TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) containing 0.1 volumes of 3 M sodium acetate, pH 5.2, then re-extracted with 0.5 volumes of phenol, followed by 0.5 volumes of chloroform/isoamyl alcohol. To remove contaminating proteoglycans, RNA was then precipitated with a high salt precipitation solution, three volumes of 4 M sodium acetate, pH 7.0, at −20 °C overnight. Total RNA was recovered by centrifugation, washed with 75% cold ethanol, air-dried, dissolved in DEPC-treated water, and stored at −70 °C until further use. RNA concentration, and the ratio of the absorbance at 260 and 280 nm, was calculated for each total RNA sample.

The trabecular bone for each time-point from the surgical cases S6, S7, and S8, used for time-course analyses of RNA integrity, were weighed prior to RNA extraction. RNA purity and the integrity of the 28S and 18S rRNA bands were assessed on ethidium bromide-stained 1% w/v agarose-formaldehyde gels.

2.4. Northern blot analysis of human GAPDH mRNA in total RNA isolated from human post-mortem and surgical trabecular bone

Total RNA isolated from human post-mortem and surgical trabecular bone (3 μg per lane) was denatured and size-separated by electrophoresis on 1% w/v agarose-formaldehyde gels. Following electrophoresis, the ethidium bromide-stained 28S (5 kb) and 18S (1.8 kb) rRNA bands were identified on the gels using a FluorImager (Molecular Dynamics, Sunnyvale, CA, USA) to assess the integrity of the RNA. The gels were transferred to a nylon membrane, and hybridised with a ^{32}P-labelled GAPDH probe (PCR-generated 415 bp DNA fragment of human GAPDH, Table 3) to detect human GAPDH mRNA (1.28 kb). Bound probe was quantified using a PhosphorImager (Molecular Dynamics) and the volume integration function of ImageQuant v.3.3 software (Molecular Dynamics). The relative intensity data of the 28S and 18S rRNA bands from ethidium bromide-stained 1% agarose-formaldehyde gels, and the GAPDH mRNA bands from Northern blots, were expressed per mg of bone tissue for the surgical cases S6 and S7. The relative half-lives, that is, the incubation time required for 28S and 18S rRNA, and GAPDH mRNA, to

Table 3
RT-PCR oligonucleotide primers and amplified PCR product sizes

Target gene	Sense	Primer sequence (5′–3′)	PCR product size (bp)	GenBank accession number
CTR	S	GCAATGCTTTCACTCCTGAGAAACT	780/732[a]	NM_001742/X69920
	AS	AGTGCATCACGTAATCATATATC		
IL-6	S	ATGAACTCCTTCTCCACAAG	544	NM_000600
	AS	GTGCCTGCAGCTTCGTCAGCA		
OCN	S	ATGAGAGCCCTCACACTCCTCG	255	NM_199173
	AS	GTCAGCCAACTCGTCACAGTCC		
OPG	S	TGCTGTTCCTACAAAGTTTACG	433	NM_002546
	AS	CTTTGAGTGCTTTAGTGCGTG		
RANK	S	CCTACGCACAAGGCGAAGATGC	702	NM_003839
	AS	CGTAGACCACGATGATGTCGCC		
RANKL	S	ATAGAATATCAGAAGATGGCACTC	665	NM_003701
	AS	TAAGGAGGGGTTGGAGACCTCG		
TGF-β1	S	CTAGACCCTTTCTCCTCCAGGAGACG	224	NM_000660
	AS	GCTGGGGGTCTCCCGGCAAAAGGT		
GAPDH	S	CATGGAGAAGGCTGGGGCTC	415	NM_002046
	AS	CACTGACACGTTGGCAGTGG		

S, sense primer; AS, antisense primer.
[a] Amplified PCR product sizes corresponding to the calcitonin receptor insert-positive and insert-negative isoforms, respectively [13].

decrease to 50% of the starting value, were derived using exponential regression analysis of the band intensity of 28S and 18S rRNA, and GAPDH mRNA, per mg bone tissue versus the storage interval (h).

2.5. Semi-quantitative RT-PCR of total RNA isolated from human post-mortem and surgical trabecular bone

cDNA was synthesized from 1 μg of total RNA from each sample, that is, four time-points from post-mortem case A18, eight time-points from surgical case S7, and seven time-points from surgical cases S6 and S8. To limit differences in the efficiency of cDNA synthesis, cDNA was synthesized from all samples from each case at the same time. cDNA was then amplified by PCR, using human-specific oligonucleotide primer pairs [11,12], to generate products corresponding to mRNA encoding human calcitonin receptor (CTR), interleukin (IL)-6, osteocalcin (OCN), osteoprotegerin (OPG), receptor activator of nuclear factor kappa B (RANK), RANK ligand (RANKL), transforming growth factor (TGF)-β1, and the housekeeping gene GAPDH. Primer sequences and PCR product sizes are shown in Table 3. The position of each amplified PCR product within the corresponding mRNA sequence is shown in Fig. 1. To allow semi-quantification of the PCR products to enable comparison of the mRNA expression between time-points, preliminary experiments were performed to ensure that the PCR amplification cycle number, for each set of primers, was within the log-linear range of amplification [11]. PCR amplification products were resolved by electrophoresis in 2% w/v agarose gels and post-stained with SYBR® Gold (Molecular Probes, Eugene, OR, USA). The relative amounts of the PCR products were determined by quantitating the intensity of bands using a FluorImager (Molecular Dynamics) and the volume integration function of ImageQuant v.3.3 software (Molecular Dynamics). The semi-quantitative RT-PCR method has been further validated by comparison with results obtained using the Taqman™ (Applied Biosystems, Foster City, CA, USA) quantitative PCR system (linear regression analysis: $r = 0.86$, $P < 0.001$) [14].

The relative amounts of the PCR products, which were used as a surrogate for the mRNA levels, are expressed as percentages of the respective mRNA level at 24 h post-mortem for the post-mortem case A18. The relative half-lives of each mRNA species analysed for case A18 were derived from the exponential regression of the percentage of mRNA at post-mortem and after each storage interval (in h). For surgical cases S6, S7, and S8, the relative mRNA expression data are expressed per mg of bone tissue. The relative half-lives of each mRNA analysed were derived from exponential regression analysis of the relative mRNA level per mg bone tissue versus the storage interval (in h). RT-PCR data were not available for the zero time-point from

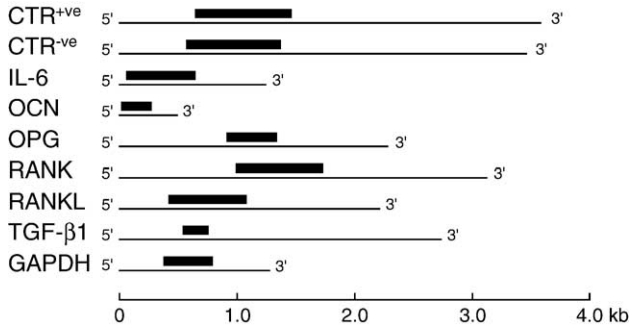

Fig. 1. Schematic representation of the position of each amplified PCR product within the corresponding mRNA sequence. Each mRNA species is represented by a line labelled 5′ to 3′. GenBank accession numbers corresponding to each mRNA species are listed in Table 3. Solid bars represent the amplified PCR products, generated using the human-specific oligonucleotide primer pairs listed in Table 3.

case S6 (bone stored at 4 °C) or the 72-h time-point from case S8 (bone stored at 37 °C) due to the small quantities of total RNA recovered.

2.6. Statistical analysis of Northern blot and RT-PCR data

Northern blot analysis was performed twice, yielding reproducible results for the quantitation of the relative density of the GAPDH mRNA bands. For each time-point, from post-mortem case A18, and surgical cases S6, S7, and S8, RT-PCR reactions were performed twice, from duplicate cDNA syntheses, which confirmed that repeated RT-PCR analysis of the same RNA samples yielded reproducible results. In addition, to minimise inter-assay variability for the comparison of time-points within a single case, all PCR products, for each case, for a given mRNA species, were electrophoresed in a single 2% agarose gel. Each data point for the statistical analyses represented the mean of two separate RT-PCR reactions. Linear and exponential regression analyses were used to examine the relationship between the relative mRNA expression levels and storage intervals of the bone, for both the Northern blot and RT-PCR analyses. Pearson's correlation coefficient was used to examine the relationship between post-mortem variables and relative intensities of the rRNA bands and GAPDH mRNA (Excel; Microsoft Corp., Redmond, WA, USA). The critical value for significance was chosen as $P = 0.05$.

3. Results

3.1. Stability of total RNA isolated from human post-mortem trabecular bone

Total RNA was isolated from femoral and iliac crest trabecular bone from 17 routine post-mortem cases with a PMI range of 4 to 84 h (Table 1). The integrity of the total RNA was assessed visually from the degradation of the 28S and 18S rRNA bands and by their relative abundance, as assessed on ethidium bromide-stained 1% agarose-formaldehyde gels (Fig. 2). RNA samples were loaded into the gel according to increasing post-mortem and storage intervals (PMIS). Although an equivalent amount of total RNA was loaded per lane (3 μg; RNA concentrations were determined by spectrophotometric assessment), considerable variability in the intensity of the rRNA bands was evident. The RNA samples were of good quality, with an average 260/280 absorbance ratio of 1.7 ± 0.2. There appeared to be a partial loss of rRNA (Fig. 2). However, the presence of intact 28S and 18S rRNA bands, with minimal smearing below the bands, is indicative of undegraded RNA in the total RNA samples. Further, when the 28S and 18S rRNA bands were quantitated, no correlation was found between the band intensities of 28S, 18S, or the 28S/18S ratio and any of the variables examined, which included the age of the individual, PMI, storage interval at 4 °C, and PMIS.

Human GAPDH mRNA was detected as a 1.28-kb band, in these post-mortem trabecular bone RNA samples, by Northern blot analysis (Fig. 2). A tailing or smearing of GAPDH mRNA below the 1.28-kb band was observed in several cases, which suggests partial degradation of the GAPDH mRNA. Interestingly, the GAPDH mRNA band was detectable up to 84 h post-mortem. The relative intensity of the GAPDH mRNA bands did not correlate with any of the variables examined, which included the age of the individual, PMI, storage interval at 4 °C, and PMIS.

A significant positive correlation was observed between the band intensities of 28S and 18S rRNA for all of the post-mortem trabecular bone RNA samples ($n = 25$; 28S vs. 18S: $r = 0.95$, $P < 0.0001$). In addition, significant positive correlations were observed between the band intensity of GAPDH mRNA and the band intensities of both 28S and 18S rRNA for all of these samples ($n = 25$; GAPDH vs. 28S: $r = 0.65$, $P < 0.0005$; GAPDH vs. 18S: $r = 0.60$, $P < 0.001$). Collectively, these observations suggest that, irrespective of the post-mortem variables for each case, there are similar relationships between the band intensities of 28S rRNA, 18S rRNA, and GAPDH mRNA. Furthermore, the

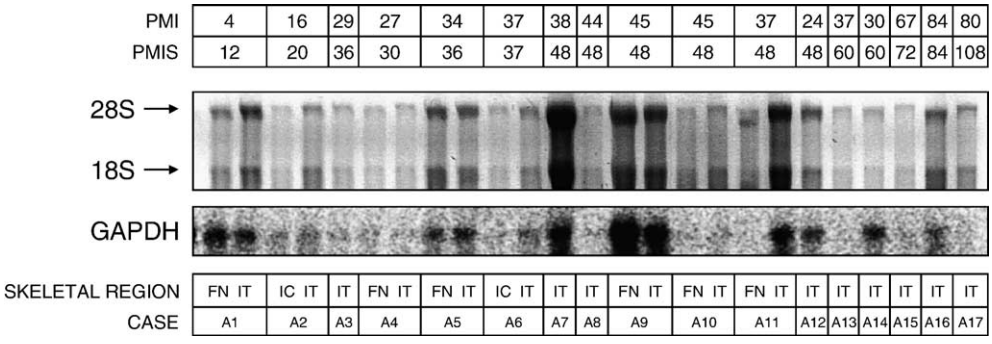

Fig. 2. Integrity of 28S and 18S rRNA and GAPDH mRNA in total RNA isolated from human post-mortem trabecular bone. RNA, 3 μg per lane, was electrophoresed through a 1% agarose-formaldehyde gel, stained with ethidium bromide to visualise the 28S (5 kb) and 18S (1.8 kb) rRNA bands, transferred to a nylon membrane, and hybridised with a ^{32}P-labelled probe to detect human GAPDH mRNA (1.28 kb). PMI, post-mortem interval (h); PMIS, sum of the post-mortem and bone tissue storage at 4 °C intervals (h); skeletal regions: FN, femoral neck; IC, iliac crest; IT, intertrochanteric region of the proximal femur; for case profiles refer to Table 1.

data suggest that the RNA stability of 28S rRNA, 18S rRNA, and GAPDH mRNA is comparable in human post-mortem trabecular bone up to 84 h post-mortem at 4 °C. When RNA was isolated from two different skeletal regions from the same post-mortem case, with the exception of case A11, significant positive correlations were observed between the two skeletal regions for the band intensity of 28S rRNA, 18S rRNA, and GAPDH mRNA ($n = 7$; 28S: $r = 0.91$, $P < 0.001$; 18S: $r = 0.74$, $P < 0.03$; GAPDH: $r = 0.98$, $P < 0.0001$), which suggests that the degradation of each of 28S rRNA, 18S rRNA, and GAPDH mRNA was similar between skeletal regions.

3.2. Stability of various mRNA species in human post-mortem trabecular bone

To investigate the effect of the PMI, or the storage interval at 4 °C, on the integrity of specific mRNA species in human post-mortem bone, total RNA was isolated from intertrochanteric trabecular bone sampled from a post-mortem case with a 24-h PMI (case A18), and at further 24-h intervals, up to 96 h, after storage of the bone tissue at 4 °C. In this case, all RNA samples had 260/280 absorbance ratios of 1.8 ± 0.1. Semi-quantitative RT-PCR was used to assess the relative abundance of mRNA encoding the osteoclast differentiation factor, RANKL; OPG, the soluble decoy receptor for RANKL; IL-6, a cytokine capable of promoting osteoclast formation; the abundant bone matrix growth factor, TGF-β1; and the housekeeping gene, GAPDH, in these RNA samples. The amount of OPG, TGF-β1, and GAPDH mRNA was observed to decline with the time the post-mortem bone was stored at 4 °C (Fig. 3). RANKL mRNA appeared to follow this pattern of decline up to 72 h, although RANKL mRNA was abundant at the 96-h time-point. IL-6 mRNA did not decline with bone storage time at 4 °C. Relative half-lives for each mRNA species analysed were derived from the exponential regression of the percentage of mRNA at 24 h post-mortem and after each storage interval (in h). The relative half-lives of OPG, TGF-β1, and GAPDH mRNA were 64, 35, and 88 h, respectively. Half-lives for RANKL and IL-6 mRNA could not be determined, as the data could not be described by exponential regression.

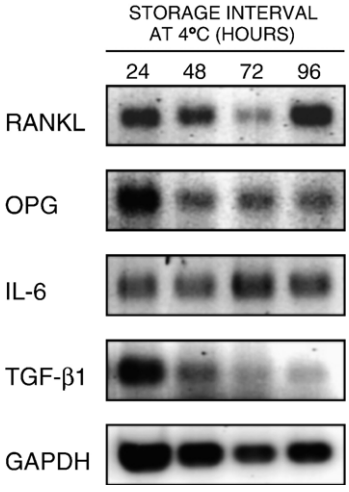

Fig. 3. Expression of RANKL, OPG, IL-6, TGF-β1, and GAPDH mRNA in total RNA isolated from post-mortem human intertrochanteric trabecular bone. Samples were from case A18 (PMI of 24 h; Table 1). Trabecular bone tissue was stored in sterile saline at 4 °C and total RNA was isolated at 24-h increments, up to 96 h, for RT-PCR analysis of specific mRNA expression. PCR products were visualised on SYBR® Gold-stained 2% agarose gels.

There was no change in the relative ratios of RANKL/GAPDH, OPG/GAPDH, and IL-6/GAPDH mRNA with bone storage time. However, the relative ratio of TGF-β1/GAPDH mRNA declined with bone storage time at 4 °C ($n = 4$; exponential regression: $r = -0.83$, $P < 0.05$), suggesting that TGF-β1 mRNA degraded at a faster rate compared to the referent mRNA, GAPDH, in this post-mortem bone. This is consistent with the relative half-life of TGF-β1 mRNA (35 h) being 2.5-fold less than for GAPDH mRNA (88 h). It is important to note that these differences in post-mortem mRNA stability do not appear to associate with the size or position of the amplified PCR fragment within the mRNA sequence (Table 3 and Fig. 1).

3.3. Effect of storage temperature and time on the stability of total RNA and various mRNA species in human surgical trabecular bone

Total RNA was isolated from femoral or tibial trabecular bone from five surgical cases (Table 2) to assess the integrity of rRNA and GAPDH mRNA in bone samples that were processed for RNA isolation immediately after their retrieval at surgery, without a storage interval. The 260/280 absorbance ratio for these surgical bone RNA samples was 1.7 ± 0.2, which was comparable to the post-mortem bone RNA samples. The assessment of rRNA integrity, on ethidium bromide-stained 1% agarose-formaldehyde gels, and GAPDH mRNA integrity, by Northern blot analysis, is shown in Fig. 4. Variability in the appearance of the 28S and 18S rRNA bands for an equivalent quantity of total RNA loaded per lane (3 μg), as observed for the post-mortem bone tissue RNA (Fig. 2), was also evident for these surgical samples. The band-tailing effect for GAPDH mRNA, which was evident for some of the post-mortem bone RNA samples (Fig. 2), was not observed for any of the surgical samples (Fig. 4). A significant positive correlation was observed between the band intensity of 28S and 18S rRNA for these surgical samples ($n = 6$; 28S vs. 18S: $r = 0.99$, $P < 0.0002$), consistent with the post-mortem bone RNA data. Surprisingly, no correlation was observed between the band intensity of GAPDH mRNA and the band intensity of either 28S or 18S rRNA for the bone samples obtained at surgery.

To investigate the effect of storage temperature and storage time on the integrity of total RNA and specific mRNA species in human bone, trabecular bone samples from the intertrochanteric region of surgical cases S6, S7, and S8 (Table 2) were used. Total RNA was isolated from

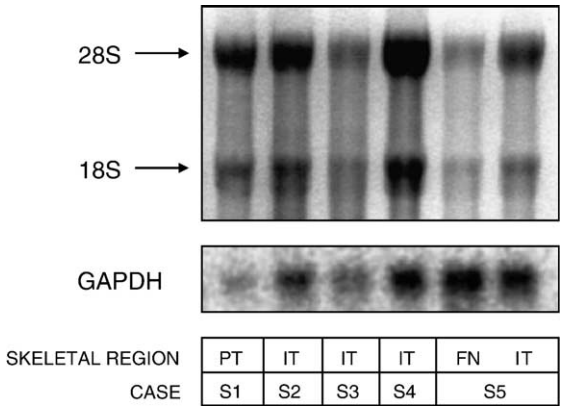

Fig. 4. Integrity of 28S and 18S rRNA and GAPDH mRNA in total RNA isolated from human surgical trabecular bone. RNA, 3 μg per lane, was electrophoresed through a 1% agarose-formaldehyde gel, stained with ethidium bromide to visualise the 28S (5 kb) and 18S (1.8 kb) rRNA bands, transferred to a nylon membrane, and hybridised with a ^{32}P-labelled probe to detect human GAPDH mRNA (1.28 kb). Skeletal regions: FN, femoral neck; IT, intertrochanteric region of the proximal femur; PT, proximal tibia; for case profiles refer to Table 2.

an eighth of each bone sample immediately after retrieval at surgery, to represent a 0-h time-point. The remaining portions were stored in sterile saline at 4 °C, or room temperature (22 ± 1 °C), or 37 °C, for cases S6, S7, and S8, respectively, and total RNA was isolated at 2, 4, 8, 16, 24, 48, and 72 h. The storage temperatures investigated were chosen because 4 °C replicates the storage temperature of the body before post-mortem examination and subsequent bone retrieval and RNA isolation; room temperature replicates the stabilised body temperature before refrigeration; and 37 °C replicates the average core body temperature before death [15], and allows the RNA degradation rate to be assessed at a temperature at which endogenous and introduced RNases are likely to be active. The total RNA yield was calculated for each time-point for each of the three cases. There was no significant decline with time in the total RNA yield or 260/280 absorbance ratio for the RNA from the bone samples stored at 4 °C or room temperature (cases S6 and S7). The mean RNA yield for bone samples stored at 4 °C (n = 8) and room temperature (n = 8) was 0.072 ± 0.038 and 0.109 ± 0.051 μg RNA/mg wet weight, respectively. However, both the total RNA yield and the 260/280 absorbance ratio declined rapidly with time for the bone samples stored at 37 °C (case S8; n = 8; exponential regression: $r = -0.86$, $P < 0.002$; $r = -0.91$, $P < 0.001$; respectively). The total RNA yield from the 37 °C stored bone samples was not sufficient for Northern blot analysis.

Total RNA integrity for the 4 °C and room temperature time-course bone RNA samples was assessed by the relative abundance of the 28S and 18S rRNA bands on ethidium bromide-stained 1% agarose-formaldehyde gels (Fig. 5). The band intensities of 28S and 18S rRNA were expressed per mg of bone tissue to correct for differences in sample tissue weight. A negative exponential correlation was observed between the band intensities of 28S rRNA, 18S rRNA, and the 28S/18S ratio, and the storage interval at 4 °C, but not at room temperature (Table 4). There may be more variability between time-points in the intensity values of 28S and 18S rRNA in bone stored at room temperature in comparison to 4 °C due to small fluctuations in the temperature during the time-course (i.e., 22 ± 1 °C; compare Fig. 5A and B). The stability of GAPDH mRNA in the 4 °C and room temperature time-course bone RNA samples was assessed by Northern blot analysis, and the band intensity values representing GAPDH mRNA were expressed per mg of bone tissue. By exponential regression, the relative abundance of GAPDH mRNA negatively correlated with the storage interval at both 4 °C and room temperature (Table 4).

The relative half-lives of 28S and 18S rRNA at 4 °C were 26 and 39 h, respectively. The 28S and 18S rRNA half-lives at room temperature could not be determined, as the data were not described by a significant exponential regression. Half-lives of GAPDH mRNA at 4 °C and room temperature

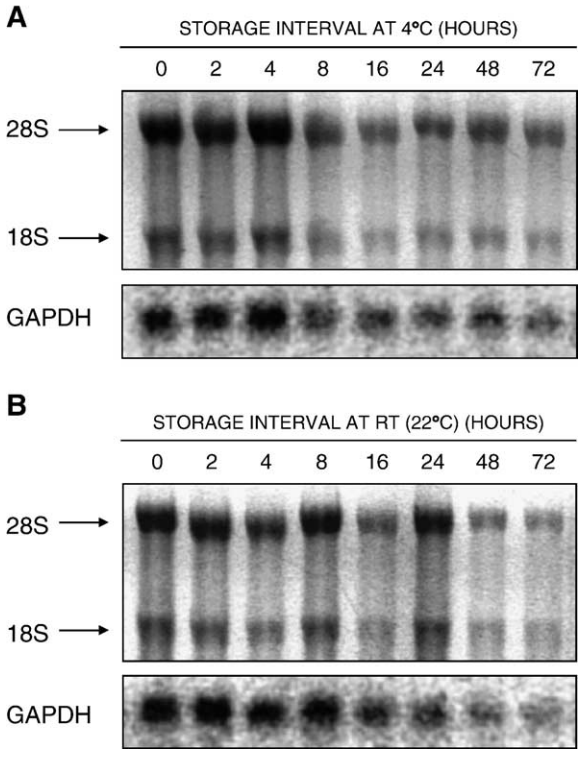

Fig. 5. Integrity of 28S and 18S rRNA and GAPDH mRNA in total RNA isolated from human surgical trabecular bone stored at 4 °C or room temperature. (A) Total RNA isolated from case S6 (Table 2). Trabecular bone tissue was stored in sterile saline at 4 °C for 0, 2, 4, 8, 16, 24, 48, and 72 h and total RNA isolated at these time-points. (B) Total RNA isolated from case S7 (Table 2). Trabecular bone tissue was stored in sterile saline at room temperature (22 °C ± 1 °C; RT) for 0, 2, 4, 8, 16, 24, 48, and 72 h and total RNA isolated at these time-points. RNA, 3 μg per lane, was electrophoresed through a 1% agarose-formaldehyde gel, stained with ethidium bromide to visualise the 28S (5 kb) and 18S (1.8 kb) rRNA bands, transferred to a nylon membrane, and hybridised with a ^{32}P-labelled probe to detect human GAPDH mRNA (1.28 kb).

Table 4
The linear and exponential correlation coefficients between the times the bone tissue was stored at 4 °C or room temperature and the levels of 28S and 18S rRNA, and GAPDH mRNA

RNA (n = 8)	Storage temperature			
	4 °C		RT (22 ± 1 °C)	
	Linear	Exponential	Linear	Exponential
28S rRNA	$r = -0.65$	$r = -0.80$	$r = -0.46$	$r = -0.46$
	$P < 0.05$	$P < 0.005$	NS	NS
18S rRNA	$r = -0.60$	$r = -0.70$	$r = -0.50$	$r = -0.52$
	NS	$P < 0.03$	NS	NS
28S/18S rRNA	$r = -0.63$	$r = -0.69$	$r = -0.19$	$r = -0.15$
	NS	$P < 0.03$	NS	NS
GAPDH mRNA	$r = -0.55$	$r = -0.65$	$r = -0.61$	$r = -0.70$
	NS	$P < 0.05$	NS	$P < 0.03$

The two-tailed P value for each correlation is given. NS, not significant; RT, room temperature.

were 48 and 39 h, respectively. Interestingly, at both 4 °C and room temperature, there was no change in the ratio of GAPDH mRNA/28S rRNA or GAPDH mRNA/18S rRNA with increasing bone storage time. This suggests that there is no difference between the degradation rates of GAPDH mRNA and rRNA in surgical trabecular bone stored in sterile saline at either 4 °C or room temperature.

Semi-quantitative RT-PCR was used to assess the relative abundance of mRNA encoding RANKL, OPG, RANK, CTR, OCN, IL-6, TGF-β1, and GAPDH, in the time-course RNA samples, where the bone, retrieved at surgery from cases S6, S7, and S8, was stored at either 4 °C, room temperature, or 37 °C, respectively. In general, the expression of these mRNA species declined significantly by 48 h in bone stored at 4 °C (Fig. 6 and data not shown). For the bone stored at room temperature, RANKL, OPG, RANK, CTR, and TGF-β1 mRNA expression had declined significantly by 24 h (data not shown). The expression of GAPDH mRNA, assessed by semi-quantitative RT-PCR for the 4 °C (case S6) and room temperature (case S7) time-courses, had only partially declined by 72 h (data not shown), in contrast with the significant exponential decline in GAPDH mRNA observed with Northern blot analysis for both the 4 °C and room temperature time-courses (Fig. 5 and Table 4). Northern blot analysis detects full-length GAPDH mRNA transcripts while the RT-PCR analysis in this study used oligonucleotide primers specific to human GAPDH mRNA, which amplify a 415-bp fragment between the exon 4/5 boundary and within exon 7 (Table 3 and Fig. 1). This RT-PCR for GAPDH mRNA would therefore amplify full-length and partially degraded GAPDH mRNA transcripts, degraded from the 3′ end, to within exon 7, and/or degraded from the 5′ end, to exon 5. The discrepancy between the Northern blot and RT-PCR analysis for the 4 °C and room temperature time-courses suggests that full-length GAPDH mRNA was partially degraded over time, with relatively much less cleavage occurring in the region of sequence being amplified by PCR.

The expression of all the mRNA species in bone stored at 37 °C (case S8) declined over time to give almost no detectable PCR product by 48 h (Fig. 6 and data not shown).

The mRNA expression data for the 4 °C, room temperature, and 37 °C time-courses were expressed per mg of bone tissue to correct for differences in tissue-sample weight and to allow the calculation of the relative half-lives of the mRNA species. Expression of OPG, CTR, OCN, and TGF-β1 mRNA declined significantly in bone stored at 4 °C, with exponential regression showing half-lives of 21, 35, 41, and 21 h, respectively. At room temperature, the expression of RANK and CTR mRNA declined significantly with an exponential regression yielding half-lives of 18 and 45 h,

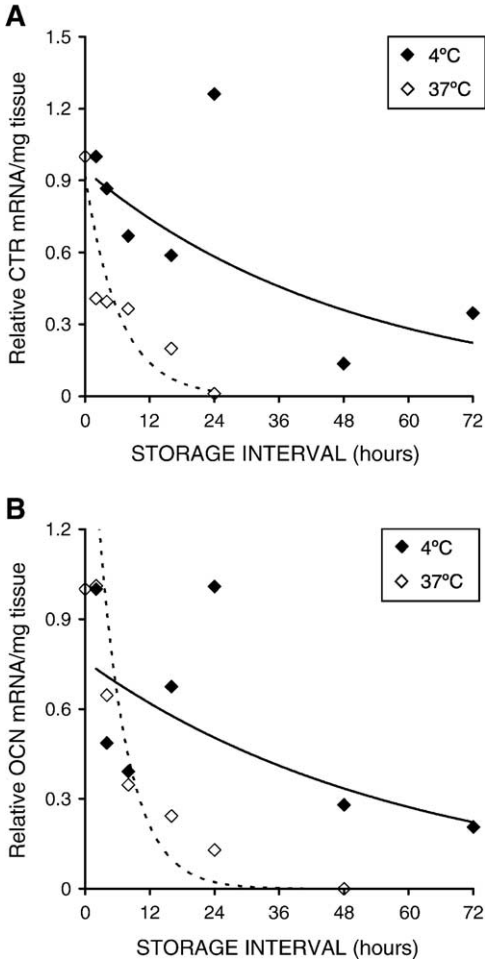

Fig. 6. Effect of storage temperature and time on stability of CTR and OCN mRNAs. Trabecular bone tissue from case S6 was stored in sterile saline at 4 °C for 2, 4, 8, 16, 24, 48, and 72 h, and from case S8 at 37 °C for 0, 2, 4, 8, 16, 24, and 48 h. Total RNA was isolated at these time-points and analysed using semi-quantitative RT-PCR. The relative value at the first time-point was set to one and values at other time-points shown relative to this point. (A) There was a significant exponential decline in the relative value of CTR mRNA with storage time at both 4 °C ($y = 0.94e^{-0.02x}$; $r = -0.69$ and $P < 0.05$) and 37 °C ($y = 0.92e^{-0.16x}$; $r = -0.92$ and $P < 0.002$). (B) There was a significant exponential decline in the relative value of OCN mRNA with storage time at both 4 °C ($y = 0.76e^{-0.02x}$; $r = -0.73$ and $P < 0.03$) and 37 °C ($y = 1.96e^{-0.19x}$; $r = -0.95$ and $P < 0.001$).

respectively. Half-lives could not be determined for RANK, RANKL, IL-6 and GAPDH at 4 °C, and RANKL, OPG, IL-6, OCN, TGF-β1 and GAPDH at room temperature as their decay could not be described by exponential regression. All of the mRNA species rapidly degraded in bone stored at 37 °C, with half-lives ranging from four (RANK and OCN) to 14 h (GAPDH). This rapid degradation of mRNA in bone stored at 37 °C is evident in Fig. 6.

Each mRNA species was also represented as a ratio of the respective PCR product/GAPDH PCR product for the 4 °C, room temperature, and 37 °C time-course surgical bone RNA samples. At 4 °C, the relative ratios of OPG/GAPDH, RANK/GAPDH, OCN/GAPDH, and TGF-β1/GAPDH mRNA declined with bone storage time ($n = 7$; exponential regression: OPG: $r = -0.93$, $P < 0.001$; RANK: $r = -0.71$, $P < 0.04$; OCN: $r = -0.84$, $P < 0.005$; TGF-β1: $r = -0.91$, $P < 0.001$), suggesting that OPG, RANK, OCN, and TGF-β1 mRNA degraded at a faster rate compared to the reference mRNA, GAPDH, in bone stored at 4 °C. At room temperature, the relative ratios of RANK/GAPDH and CTR/GAPDH mRNA also declined with bone storage time ($n = 8$; exponential regression: RANK: $r = -0.72$, $P < 0.03$; CTR: $r = -0.67$, $P < 0.05$), again suggesting that RANK and CTR mRNA degraded at a faster rate compared to GAPDH mRNA under these conditions. The relative ratio of all of the mRNA species/GAPDH mRNA declined significantly with bone storage time at 37 °C, suggesting that the degradation rate of all of these mRNA species was faster than GAPDH mRNA at this temperature.

4. Discussion

To determine the reliability of gene expression studies in human post-mortem bone, it is important to evaluate the stability of RNA isolated from such tissues as a function of the post-mortem interval. This study examined the stability of total RNA and bone-specific mRNA species in bone samples obtained from routine autopsies and at surgery. Total RNA can be reliably isolated from human bone obtained at post-mortem and surgery if the following conditions are adhered to: cases are selected with a post-mortem interval of less than 48 h, the time that bone is stored after retrieval at autopsy or surgery is minimised, and the optimal temperature for any storage and transport of the bone before RNA isolation is 4 °C.

The overall degradation of total RNA isolated from trabecular bone from 17 post-mortem cases was assessed by the integrity of the 28S and 18S rRNA bands on ethidium bromide-stained agarose-formaldehyde gels, and GAPDH mRNA levels assessed by Northern blotting. Although we found a loss of ribosomal RNA (28S and 18S), which is indicative of degradation, in some post-mortem cases, there was in general no correlation between the relative rRNA band intensities, or GAPDH mRNA band intensity, and PMI up to 84 h. These observations are consistent with the report of well-preserved RNA in rabbit connective tissues (ligament, cartilage, and tendon) in carcasses stored at 4 °C for up to 96 h post-mortem [16], and suggest that, at low temperature, cellular RNases are either maintained in sequestered intracellular sites or maintained in an inactive state up to 96 h post-mortem. mRNA half-lives in vivo vary from a few minutes for labile (relatively unstable and transient) species, such as cytokines, up to 24 h or more for stable species, such as the globins [17]. However, stability studies of specific mRNA transcripts from human post-mortem tissues, predominantly neurological, have shown that mRNA stability post-mortem is not dependent on the in vivo half-life of the mRNA [6,18,19]. For example, Yasojima et al. [18] reported a similar mRNA degradation rate between the highly stable housekeeping gene cyclophilin, and the highly labile COX-2 mRNA, which has a half-life of 30 min in vivo, in human post-mortem brain tissue obtained up to 96 h. Furthermore, these authors proposed that the in vivo pathways of mRNA degradation, for each mRNA in pre-mortem degradation, would occur but at a slower rate post-mortem. Consistent with this concept, in the present study mRNA species with different half-lives in vivo, ranging from 15 min for IL-6 up to 6 h for TGF-β1 [20,21], were all detectable by RT-PCR analysis in RNA isolated from trabecular bone obtained 24 h post-mortem.

In our study, although there was no correlation between rRNA degradation and the PMI, there was considerable variability in the quality of the RNA recovered between cases with differing PMIs, which may be due to variability between the cases in a number of unrecorded pre-mortem and post-mortem variables such as the time between death and storage of the body at 4 °C in the mortuary, and different body cooling rates due to different medical conditions prior to death [22,23]. In addition, the body-cooling rate after death will be affected by the individual's physique and build [23]. The PMI also depends on a number of uncontrolled factors, such as the time of day that the individual died, how quickly consent can be obtained from the next-of-kin for tissue donation, or if the individual died in the hospital: if the body needs to be transported between institutions the PMI can be lengthened by 1 or 2 days [23].

The Northern blot of GAPDH mRNA for a selection of bone RNA samples from human post-mortem cases showed a tailing effect below the 1.28-kb GAPDH mRNA band, which is suggestive of partial GAPDH mRNA degradation. This tailing effect was also observed for GAPDH mRNA on Northern blots of rabbit post-mortem connective tissue RNA [16], and a similar effect was observed for mRNA species in human post-mortem brain tissues [24,25] and soft tissues associated with human temporal bones [6]. Tong et al. [26] used competitive RT-PCR to show that specific mRNA species were degraded to an extent similar to the degradation of the total RNA, suggesting that the expression of specific mRNAs in

samples with degraded total RNA could be normalised to a constitutively expressed gene to allow correct quantification of relative mRNA levels and permit comparison of gene expression in RNA samples of varying integrity. In our study, the relative expression of mRNA species corresponding to the skeletally active molecules RANKL, OPG, RANK, IL-6, CTR, OCN, and TGF-β1 declined in bone stored at 4 °C (by 48 h), room temperature (by 24 h), and 37 °C (by 6 h). The relative ratio of each specific mRNA/GAPDH mRNA was analysed as a function of bone storage time at each temperature. At 4 °C and room temperature, some mRNA species appeared to degrade at a faster rate than the referent mRNA, GAPDH, while at 37 °C, the degradation rate of all of the mRNA species appeared to be faster than for GAPDH mRNA. Therefore, given that there appear to be differences in mRNA degradation rates in human bone, it is important that the storage interval before RNA isolation from bone retrieved post-mortem or at surgery is kept to a minimum. Furthermore, the expression of specific mRNA species in human post-mortem and surgical bone should be normalised to GAPDH mRNA expression to reduce the impact of bone sampling time. Ideally, the relationship between storage time and reduction in mRNA levels should be established for particular genes of interest to validate their comparison between autopsy and surgically obtained samples.

The RNA degradation seen in the stored surgical bone may be due to the introduction of exogenous RNases during the processing of the tissue and/or additional endogenous RNases passing into solution with longer storage times. The samples used for the time-course analyses were divided and each sample separately stored at the study temperature in sterile saline. An alternative approach would be to retain the tube saw bone sample intact in sterile saline and cut or section contiguous pieces of bone as each time-point arises. This approach may result in less initial exposure of the tissue to potential exogenous RNases. In addition, the mechanical action and/or generation of heat from cutting may damage cell membranes at the borders of the tissue sample, resulting in the release of lysosomal RNases into the storage solution, which may explain the rapid mRNA degradation observed for surgical bone stored at 37 °C, an optimal activity temperature for RNases. Similarly, freezing and thawing tissues may result in the release of RNases from lysosomes [18,24]. Therefore, minimising lysosomal damage and subsequent RNase release should minimise the rapid degradation of RNA in post-mortem tissues.

Total RNA isolated from different skeletal sites from human post-mortem cases shows signs of partial degradation up to 84 h post-mortem at 4 °C, indicating that RT-PCR analysis is the preferred technique for the analysis of gene expression in human post-mortem bone as it tolerates partial RNA degradation. In addition, the analysis of a post-mortem bone sample with time at 4 °C demonstrated that bone-specific mRNAs begin to degrade after 24 h of storage. Therefore, for gene expression studies in bone, post-mortem cases with a PMI of less than 48 h should be selected, and the time the bone is stored at 4 °C after retrieval at autopsy should be kept to a minimum. In addition, processing of the post-mortem bone should be controlled to minimise introduction of exogenous RNases. The optimal temperature for any storage and transport of the bone before RNA isolation is 4 °C. If RNA is isolated from pathological bone tissues retrieved from surgery, this bone should be processed and handled as for post-mortem bone, with the optimal temperature for any short-term storage being 4 °C. The data presented in this study show a 50% reduction by 24 h in the relative abundance of some bone-specific mRNAs in surgical bone tissues stored at 4 °C. This suggests that surgical bone tissues should be processed within 12 h of retrieval for the reliable isolation of intact total RNA. Furthermore, once any sectioning or cutting of the bone has occurred the samples should be immersed into a strong denaturant buffer to inactivate any endogenous RNases released. When using human post-mortem and surgical bone for gene expression analyses, it is recommended that initial studies be performed to assess the stability of individual mRNA species in bone stored at 4 °C.

If these conditions are adhered to, RNA can be reliably isolated from human post-mortem and surgical bone to study ex vivo the pattern of gene expression in healthy individuals and in patients with musculoskeletal diseases such as osteoporosis and osteoarthritis. Indeed, using these criteria for the isolation of RNA from human bone, we have reported differential mRNA expression of osteoclastogenic factors in fragility hip fracture and osteoarthritic femoral bone, in comparison to skeletal site-matched post-mortem bone [11,14,27]. Furthermore, we have provided validation of this procedure by showing that histomorphometric indices of bone remodelling tightly associated with mRNA expression of a key osteoclast differentiation factor, RANKL, relative to expression of the decoy receptor for RANKL, OPG, in human post-mortem femoral bone [27]. Studies that map gene expression to bone morphology can provide significant insight into the molecular basis for altered bone tissue architecture associated with ageing and pathology.

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